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A possible role for saprotrophic microfungi in the N nutrition of ectomycorrhizal *Pinus resinosa*

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Abstract

We determined whether *Pinus resinosa*, selected ectomycorrhizal and saprotrophic microfungi have access to various organic nitrogen sources commonly found in the forest. Vector analysis demonstrated nitrogen limitation of the *P. resinosa* in the plantation from which most of the fungi were isolated, establishing this study's relevance. Nonmycorrhizal *P. resinosa* seedlings did not absorb significant N from amino acids. The ectomycorrhizal fungi, including *Pisolithus tinctorius*, *Suillus intermedius* and *Tylopilus felleus*, obtained substantial N from amino acids, a limited amount of N from glucosamine, and essentially no N from protein–tannin complex. In contrast, *Penicillium* and *Trichoderma* readily acquired N from protein–tannin and glucosamine. Thus, there was an increasing ability to obtain N from complex organic N sources from plant to ectomycorrhizal fungi to saprotrophic fungi. Furthermore, N mineralization from an organic N source by *Penicillium* depended on the C:N ratio. We conclude that acquisition of relatively simple organic N sources by *P. resinosa* is likely to be largely indirect via ectomycorrhizal fungi, and that more complex organic N sources may become accessible to ectomycorrhizal fungi (and thus possibly their host plants) following mineralization by saprotrophic fungi such as *Penicillium* or *Trichoderma* when C:N ratios are sufficiently low.

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1. Introduction

Nitrogen (N) is a major limiting resource for plant growth in many temperate forest ecosystems (Gosz, 1981; Aber et al., 1989; Stump and Binkley, 1992). In such ecosystems the largest pool of N is typically organic, occurring within living organisms and as components of detritus in the forest floor (Johnson, 1992). Amino acids and proteins are among the most abundant forms of organic N in the soil (Grov, 1963a,b; Sowden and Ivarson, 1966; Abuarghub and Read, 1988; Turnbull et al., 1996; Schulten and Schnitzer, 1998; Johnsson et al., 1999). Amino sugars may also comprise a significant fraction of organic nitrogen in soils (Johnsson et al., 1999; Milchalzik and Matzner, 1999; Rodionov et al., 2001; Dai et al., 2002; Turrión et al., 2002; Praveen-Kumar et al., 2002; Xu et al., 2003) because fungi, including ectomycorrhizal fungi, contribute a very large fraction of microbial biomass in forest ecosystems (Högberg and Högberg, 2002) and because the cell walls of fungi contain large concentrations of chitin, a polymer of the amino sugar acetyl glucosamine.

Nitrogen mineralization is conventionally thought to be necessary to convert organic N into forms that are available to plants (Myrold, 1998), but recently there has been considerable discussion about the importance of mineralization in N cycling (Chapin, 1995; Kaye and Hart, 1997). There are three major reasons for this. First, some plant species are themselves able to absorb simple organic N compounds such as amino acids (Chapin et al., 1993; Kielland, 1994; Schimmel and Chapin, 1996; Turnbull et al., 1996; Näsholm et al., 1998), although this

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phenomenon is by no means universal (Stribley and Read, 1980). Second, some ECM fungi may be able to transfer N to their hosts after absorbing from the forest floor simple organic compounds such as amino acids or small peptides (Abuzinadah et al., 1986; Turnbull et al., 1995, 1996), which route was probably first suggested by Frank (1894). It should be noted, however, that there is considerable interspecific and intraspecific variability among ECM fungi in their ability to do this (Abuzinadah et al., 1986; Keller, 1996; Sawyer et al., 2001). Third, some ECM fungi have the ability to acquire some substances, notably phosphate, directly from basidiomycete, wood-decaying fungi (Lindahl et al., 1999). Lindahl et al. (2002) suggest that such transfer occurs via 'combative interactions' between ECM and wood decay fungi. These combative interactions could potentially lead to transfer of N as well as P.

In contrast with wood decaying fungi, the common soil microfungi may interact with ECM fungi in a different manner owing to their inability to form rhizomorphs and thus their inability to translocate resources over great distances. This inability could lead to local carbon limitations (low C:N ratios) and the resultant mineralization of N (Lindahl et al., 2002). If the saprotrophic microfungi were able to mineralize N from organic sources that are less directly accessible to ectomycorrhizal fungi, one might predict the existence of either additive or synergistic interactions between coexisting microfungi and ectomycorrhizal fungi in terms of host plant N or P content. Indeed, previous microcosm experiments of ours showed that saprotrophic organisms in the forest floor could act additively or synergistically with an ectomycorrhizal fungus (Pisolithus tinctorius) to influence N or P contents of P. resinosa seedlings (Koide and Kabir, 2001). We were also able to show that saprotrophic microfungi isolated from a P. resinosa plantation could mineralize protein–tannin complex (Wu et al., 2003). Our objective in the present research was to compare common saprotrophic microfungi and ectomycorrhizal fungi in their ability to acquire N from organic sources using a broader range of both ectomycorrhizal fungi and organic N sources. Moreover, we wanted to determine the dependence of mineralization of organic N by common saprotrophic microfungi on the C:N ratio.

The soils of many temperate forests typically contain large concentrations of phenolic compounds, including humic acids and tannins (Coulson et al., 1960; Kuiters and Sarink, 1986), and their presence can strongly affect the capacity of at least some ECM fungi to utilize organic forms of N. This can occur either because of their direct effects on fungal physiology (Koide et al., 1998), or indirectly by interaction with the organic N compounds (Horner et al., 1988; Bending and Read, 1996). Naturally occurring tannins form hydrogen bonds with proteins, causing them to precipitate (Hagerman, 2002). In addition, phenolic compounds may interact with various compounds without causing them to precipitate (Qualls et al., 1991). The effects of interactions among phenolic compounds and organic N sources in the forest floor on the availability of N to saprotrophic and ECM fungi are poorly understood, but they may be important when considering whether organic N sources are directly or even indirectly available to plants. Therefore in some of our tests we combined tannin with the organic N source to determine its effect on the bioavailability of N.

2. Materials and methods

2.1. Study 1. Vector analysis of plantation trees

This study was carried out in an approximately 65-year-old plantation of P. resinosa Ait (red pine), located in State College, Centre County, PA, USA, and previously described in Koide et al. (2000). Vector analysis is a method of determining nutrient deficiencies by analyzing the effect of fertilizer application on the nutritional status and weight of leaves (Weetman, 1989; Kiefer and Fenn, 1997). We established four treatments on 29 October 1997 including nitrogen (N), phosphorus (P), dolomitic lime, and control, and these were arranged in a complete block design with eight replications. Within each block four trees were selected for uniformity. The average distance between selected trees within a block was 17.4 m. Treatments were randomly assigned to the selected trees. At least one untreated tree separated trees of the various treatments. All treatments were applied to the forest floor in a 2.25 m radius around an individual tree (16 m^2). N was applied as type 120R, 4-month, controlled release urea (86% urea, 14% coating, ExxonMobil Research and Engineering, Houston, TX, USA) at 500 kg N ha^{-1}. P was applied as triple super phosphate (0-46-0, Setre, Inc., Hazelton, PA, USA) at 500 kg P ha^{-1}. Dolomitic lime (White Stone Co., Paradise, PA, USA) was applied at 3570 kg lime ha^{-1}. The control trees received no amendment.

On 27 October 1998, a branch from the upper region of each tree (receiving direct sunlight) was clipped using a pole pruner with extensions. Needles were taken from each branch. The weight of 100 fascicles was determined following drying at 70 °C. All samples were then ground, and subsamples were digested at 400 °C in a 1:1 mixture of concentrated H_2SO_4 and 30% H_2O_2. N concentrations were determined using the Nessler method (Jensen, 1962). P concentrations were determined using the molybdo-phosphate method of Watanabe and Olsen (1965). Ca and Mg concentrations were determined by atomic absorption spectrometric methods (Richards, 1993).

2.2. Study 2. Potential N sources for red pine seedlings

Seeds of red pine were obtained from F.W. Schumacher Co. (Sandwich, MA, USA). They were surface-sterilized in

30% H_2O_2 for 30 min and rinsed in sterile, distilled H_2O. Seeds were then sterilely transferred to a gelled nutrient medium in petri dishes to check for contaminating microorganisms during germination. All contaminated seeds were rejected. The medium for seed germination contained: 0.5 g l^{-1} KH_2PO_4, 0.25 g l^{-1} $MgSO_4 \cdot 7H_2O$, 0.025 g l^{-1} NaCl, 0.05 g l^{-1} $CaCl_2$, 0.5 g l^{-1} NH_4Cl, 8 mg l^{-1} NaFeEDTA, 0.75 mg l^{-1} KI, 6 mg l^{-1} $MnCl_2 \cdot 4H_2O$, 2.6 mg l^{-1} $ZnSO_4 \cdot 7H_2O$, 1.5 mg l^{-1} H_3BO_4, 0.13 mg l^{-1} $CuSO_4 \cdot 5H_2O$, 0.0024 mg l^{-1} $Na_2MoO_4 \cdot 2H_2O$, 0.5 mg l^{-1} nicotinic acid, 0.1 mg l^{-1} thiamine HCl, 0.1 mg l^{-1} pyridoxine HCl, 3.0 mg l^{-1} glycine, 2.5 g l^{-1} malt extract, 0.125 g l^{-1} yeast extract, 10 g l^{-1} glucose, and 12 g l^{-1} agar.

Sterile, germinated seedlings were sterilely transplanted into 160 ml glass growth tubes (40×130 mm^2) filled with 65 ml of a sterile (autoclaved) 6:4 mixture of vermiculite and perlite moistened with 26 ml of a sterile nutrient solution similar to that above, but without glucose, agar, malt extract, yeast extract or NH_4Cl. Seedlings were randomly assigned to one of four different treatments including control (4 mg N l^{-1} as NH_4Cl, also referred to as base N), amino acids (base N and 36 mg N l^{-1} as Casitone (0259-15, Difco Co., Detroit, MI, USA), equivalent to 270 mg l^{-1} casitone), amino acids with tannin (base N and 36 mg N l^{-1} as Casitone and 270 mg l^{-1} tannic acid), and NH_4^+ (40 mg N l^{-1} as NH_4Cl). Thus, the total N available to each control plant was 104 μg N and the total N available to each amino acid or amino acid–tannin plant was 1040 μg N. Casitone is a mixture of amino acids containing 13.3% total N. Its amino acid composition (%) is as follows: alanine (3), arginine (4), aspartic acid (7), glutamic acid (20), glycine (2), histidine (2), isoleucine (4), leucine (9), lysine (14), methionine (2), phenylalanine (4), proline (9), serine (5), threonine (4), tryosine (2), valine (4). There were eight replicate seedlings per treatment. After 97 d of growth seedlings were harvested, dried at 70 °C, and weighed.

2.3. Study 3. Potential N sources for ectomycorrhizal fungi

Six species of ectomycorrhizal fungi were used in this study. Isolates of *Amanita rubescens* Pers., *Scleroderma citrinum* Pers., *Suillus intermedius* (Smith & Thiers) Smith & Thiers, and *Tylopilus felleus* (Bull. Ex Fr.) Karsten were obtained from sporocarps collected in the red pine plantation (see Study 1). For purposes of comparison with more frequently studied ectomycorrhizal fungal species, an isolate of *P. tinctorius* (Pers.) Coker & Couch was obtained from Dr Michael Kiernan (Plant Health Care, Inc., Pittsburgh, PA, USA) and an isolate of *Cenococcum geophilum* Fr. was obtained from Dr James Trappe (USDA Forest Service, Corvallis, OR, USA). All ectomycorrhizal fungi were maintained on the gelled nutrient medium listed for Study 2.

Experiment 3-1. The ability of three of the ectomycorrhizal fungi, *P. tinctorius*, *S. intermedius* and *T. felleus* to obtain N from a mixture of amino acids was tested on the gelled nutrient medium listed for Study 2, but without malt or yeast extracts. The four N treatments in Study 2 were used. The petri dishes were 60 mm in diameter and held approximately 12 ml of medium. Thus, each fungus in the control treatment had available to it a total of 48 μg N and each fungus given amino acids or amino acids–tannin had available to it 480 μg N. There were five replicate petri dishes per fungal species. All species were harvested when the rate of increase in diameter began to markedly decline. Thus *P. tinctorius*, *S. intermedius* and *T. felleus* were harvested after 50, 50, and 51 d, respectively. Fungal colonies were harvested by melting the agar in boiling water and collecting the fungal material on a nylon mesh. Fungal material was dried at 65 °C and weighed. N concentrations were determined as in Study 1.

Experiment 3-2. The abilities of three of the ectomycorrhizal fungi, *P. tinctorius*, *S. intermedius* and *T. felleus* to obtain N from glucosamine–HCl were determined on the gelled nutrient medium listed for Study 2, but without malt or yeast extracts. The experiment was essentially the same as Experiment 3-1, including four different N treatments, but with a change of N source. In place of amino acids, glucosamine–HCl (Sigma G-4875) was used at the same N concentration (36 mg N l^{-1} as glucosamine–HCl, equivalent to 554 mg l^{-1} glucosamine–HCl). The petri dishes were 60 mm in diameter and held approximately 12 ml of medium. Thus, each fungus in the control treatment had available to it a total of 48 μg N and each fungus given glucosamine–HCl or glucosamine–HCl–tannin had available to it 480 μg N. There were five replicates for each ectomycorrhizal fungus. All species were harvested when the rate of increase in diameter began to markedly decline. Thus *P. tinctorius*, *S. intermedius* and *T. felleus* were harvested after 49, 42, and 50 d, respectively. Harvesting and nutrient analysis were performed as in Experiment 3-1.

Experiment 3-3. The ability of three of the ectomycorrhizal fungi, *P. tinctorius*, *S. intermedius* and *T. felleus* to obtain N from acetyl glucosamine was tested on the gelled nutrient medium listed in Study 2, but without malt or yeast extracts. The experiment was essentially the same as Experiments 3-1 and 3-2, but acetyl glucosamine (Sigma A-8625) was used as the N source at the same N concentration (36 mg N l^{-1} as acetyl glucosamine, equivalent to 571 mg l^{-1} acetyl glucosamine). The petri dishes were 60 mm in diameter and held approximately 12 ml of medium. Thus, each fungus in the control treatment had available to it a total of 48 μg N and each fungus given acetyl glucosamine or acetyl glucosamine–tannin had available to it 480 μg N. There were five replicates for each ectomycorrhizal fungus. All species were harvested when the rate of increase in diameter began to markedly decline. *P. tinctorius*, *S. intermedius* and *T. felleus* were harvested after 50, 50, and 51 d, respectively. Harvesting and nutrient analysis were performed as in Experiments 3-1 and 3-2.

Experiment 3-4. The ability of six ectomycorrhizal fungi, *A. rubescens*, *C. geophilum*, *P. tinctorius*, *S. citrinum*, *S. intermedius* and *T. felleus*, to obtain N from free protein or protein–tannin complex was tested. All fungi were grown on the gelled nutrient medium listed in Study 2, but without malt or yeast extracts. Also, we replaced the NaFeEDTA with $FeCl_3$ because of the possible inhibitory effects of EDTA on the activity of certain proteases (Rodier et al., 2001). The four different N treatments were: control (7 mg N l^{-1} as arginine), protein (7 mg N l^{-1} as arginine and 80 mg N l^{-1} as bovine serum albumen, Sigma A-7906), protein–tannin complex (7 mg N l^{-1} as arginine and 80 mg N l^{-1} as bovine serum albumen, equivalent to 500 mg bovine serum albumen l^{-1}, and 500 mg tannic acid l^{-1}) and tannin control (7 mg N l^{-1} as arginine with 180 mg tannic acid l^{-1}, Sigma T-0125 or T-8406). The petri dishes were 60 mm in diameter and held approximately 12 ml of medium. Thus, each fungus in the control or tannin treatments had available to it a total of 84 µg N and each fungus given protein or protein–tannin had available to it 1040 µg N. Filter-sterilized tannic acid solution and filter-sterilized bovine serum albumen were added to autoclaved nutrient medium to obtain these concentrations. A 180 mg l^{-1} tannic acid concentration was approximately equivalent to the free tannic acid concentration in the above solution of protein–tannin complex in water at room temperature. There were eight replications for each treatment of *C. geophilum* and *P. tinctorius*, and five replicates for each of the other four species. All species were harvested when the rate of increase in diameter began to markedly decline. Thus, *A. rubescens*, *C. geophilum*, *P. tinctorius*, *S. citrinum*, *S. intermedius* and *T. felleus* were harvested after 45, 55, 45, 45, 38 and 40 d, respectively. Fungal N concentrations were analyzed as above.

2.4. Study 4. Potential N sources for saprotrophic fungi

Saprotrophic fungi were isolated in October 2000 from the litter and F-layer of the *P. resinosa* plantation utilized in Study 1. To isolate saprotrophic fungi from dead pine needles from the litter, we used the common plating method (Warcup, 1950) either without washing to obtain microbes primarily on the exterior of the pine needles, or following washing in 0.005% Aerosol OT solution (Fisher #A-345) to obtain primarily microbes from the interior of the dead needles (Aoki et al., 1992). To isolate saprotrophic microbes from F-layer we plated F-layer material without having first washed it. Fungi were isolated on 1% malt extract agar consisting of 10 g l^{-1} malt extract and 12 g l^{-1} agar (pH adjusted to 4.0). Two of the saprotrophic fungi that were most frequently isolated from both needles and F-layer were *Penicillium* sp. and *Trichoderma* sp., identified on the basis of spore anatomy and sequence of the ITS portion of the rRNA gene region. One isolate of each species was used in this study.

Experiment 4-1. This experiment was carried out to test for the ability to obtain N from protein or protein–tannin complex. The experiment was performed on gelled medium in petri dishes in the same manner as in Experiment 3-4. Each fungus in the control treatment had available to it a total of 84 µg N and each fungus given protein or protein–tannin had available to it 1040 µg N. There were five replicates of each isolate—treatment combination. *Penicillium* and *Trichoderma* were harvested after 10 and 20 d, respectively. Fungal N concentrations were analyzed as above.

Experiment 4-2. This experiment was carried out to test for the ability of *Trichoderma* and *Penicillium* to obtain N from glucosamine–HCl. There were four different N sources used as in Experiment 3-2, including control, glucosamine–HCl, glucosamine–HCl with tannin, and NH_4^+. Each fungus in the control treatment had available to it a total of 48 µg N and each fungus in the other treatments had available to it 480 µg N. There were five replicates of each isolate—treatment combination. Fungi were harvested after 36 and 33 d, respectively, for *Trichoderma* and *Penicillium*. Fungal N concentration was determined as above.

Experiment 4-3. This experiment was carried out to test for the ability of *Trichoderma* and *Penicillium* to obtain N from acetyl glucosamine. The treatments were the same as in the previous experiment, but acetyl glucosamine (Sigma A-8625, 36 mg N l^{-1} as acetyl glucosamine) was used in place of the glucosamine–HCl. The petri dishes were 60 mm in diameter and held approximately 12 ml of medium. Thus, each fungus in the control treatment had available to it a total of 48 µg N and each fungus given acetyl glucosamine or acetyl glucosamine–tannin had available to it 480 µg N. There were five replicates of each isolate—treatment combination. Fungi were harvested 30 and 35 d, respectively, for *Trichoderma* and *Penicillium*. Fungal N concentration was determined as above.

Experiment 4-4. This experiment was carried out to test for the ability of the isolate of *Penicillium* to mineralize N from protein–tannin. We had previously established that little nitrification occurred under the experimental conditions, so we quantified only NH_4^+ production. Protein–tannin complex was produced as above, but the final concentrations of each were 500 mg l^{-1} and the base solution consisted of the nutrient solution listed in Experiment 3-4, but without NH_4^+ or agar. We varied the glucose concentration to adjust the C:N ratio to 12:1, 30:1 and 50:1. We then added 50 ml aliquots of the solutions to 150 ml culture flasks and inoculated with the *Penicillium* isolate. For each C:N ratio there were a total of 15 flasks, representing three replicate flasks for each of five harvest dates (10, 20, 30, 40 and 50 d after inoculation). Suspensions were filtered ultimately through 0.2 µm membrane filters prior to analysis for NH_4^+ concentrations using the salicylate method of the Hach Company (Hach, 1992). Hyphae were collected on filter paper and rinsed in water. Thereafter they were dried, weighed, and analyzed for N concentration as above.

Table 1
Mean (s.e.m.) needle dry weights 12 months following treatment of red pine in the vector analysis (Study 1)

Treatment	Needle dry weight (g 100 fascicles^{-1})
Control	6.063 (0.315)[b]
Nitrogen	7.408 (0.311)[a]
Phosphorus	7.394 (0.499)[a]
Lime	7.041 (0.479)[ab]

Treatments included control, nitrogen (urea), phosphorus (super phosphate) and dolomitic lime. $n=8$. Different letters indicate that means are significantly ($p \leq 0.05$) different from each other.

2.5. Statistical analysis

Data were analyzed using the analysis of variance module of Statgraphics (STSC, 1991). Treatment means were separated by Fisher's protected LSD method.

3. Results

3.1. Study 1. Vector analysis of plantation trees

Compared to control trees, N fertilization (with urea) significantly increased needle dry weight (Table 1), needle N concentration and N content (Fig. 1a). P-fertilization significantly increased needle dry weight (Table 1) and needle P content, but not needle P concentration (Fig. 1b). Treatment with dolomitic lime did not significantly influence needle dry weight (Table 1) or needle Ca or Mg concentrations or contents (Figs. 1c and d). According to Valentine and Allen (1990) this indicates that the trees were limited by N and P, but not by Ca or Mg.

3.2. Study 2. Potential N sources for red pine seedlings

The only N source that resulted in a significant increase in red pine seedling dry weight was NH_4^+ (Fig. 2). Neither the amino acids, nor amino acids with tannic acid resulted in a significant increase in growth over control plants.

3.3. Study 3. Potential N sources for ectomycorrhizal fungi

Experiment 3-1. Both *Pisolithus* (Pt) and *Suillus* (Sui) were able to use amino acids, amino acids with tannin, and NH_4^+ as N sources (Fig. 3a). *Tylopilus* (Tyl) could use amino acids and NH_4^+, but amino acids with tannin resulted in no fungal growth. Tannin did not have an especially growth-depressive effect on either Pt or Sui.
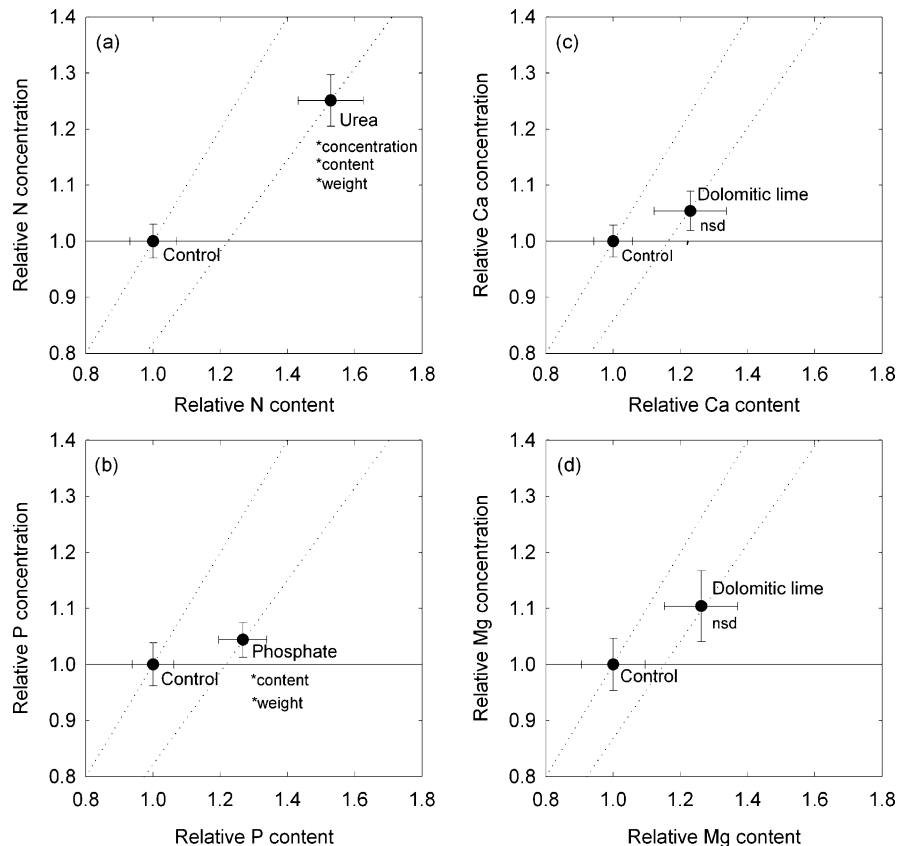

Fig. 1. Vector analysis diagrams (Study 1) including control trees and those fertilized with (a) urea, (b) phosphate, (c) and (d) dolomitic lime. *X* and *Y* axes are, respectively, relative nutrient content and concentration of needles taken from the trees. Dotted lines are isopleths of equal needle dry weight. $n=8$. * indicates a significant difference between control and treatment trees for the listed trait, either nutrient concentration of the needles, nutrient content of the needles or needle weight. nsd indicates no significant difference between control and treatment trees in both axes. Vertical and horizontal bars represent ± 1 s.e.m.
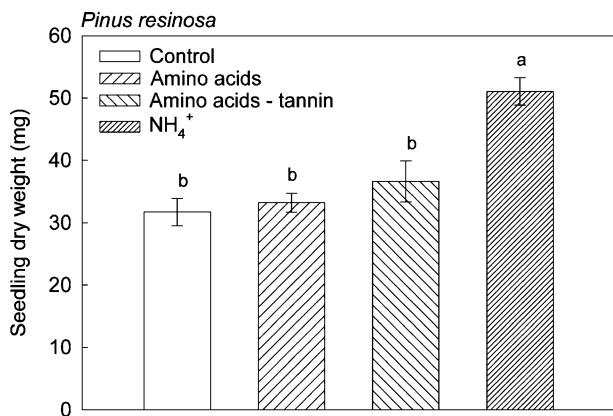
Fig. 2. Mean dry weight of seedlings of *P. resinosa* (red pine) from Study 2 given one of four N treatments including control, N as amino acids, N as amino acids and tannin, or N as NH_4^+. $n=8$. Vertical bars represent ± 1 s.e.m. Different letters indicate that means are significantly ($p \leq 0.05$) different from each other.

Experiment 3-2. Again, Pt, Sui and Tyl were each able to use NH_4^+ as an N source (Fig. 3b). Only Tyl was able to significantly utilize glucosamine–HCl as an N source, but it was a poorer N source than NH_4^+. When tannin was added to the glucosamine–HCl, Tyl colonies did not grow. Tannin did not have an especially growth-depressive effect on either Pt or Sui.

Experiment 3-3. For Pt, Sui and Tyl, colonies given acetyl glucosamine contained significantly more N than control colonies (Fig. 3c). However, there was variability among species in this regard. Tyl was the most effective in using acetyl glucosamine. In fact, Tyl N content was as high with acetyl glucosamine as with NH_4^+. Acetyl glucosamine was inferior to NH_4^+ as an N source for Pt and Sui. The addition of tannin to acetyl glucosamine had a significantly depressive effect on N content of Pt and Tyl, but not Sui.

Experiment 3-4. The six species of ectomycorrhizal fungi could each utilize bovine serum albumen (a protein) as an N
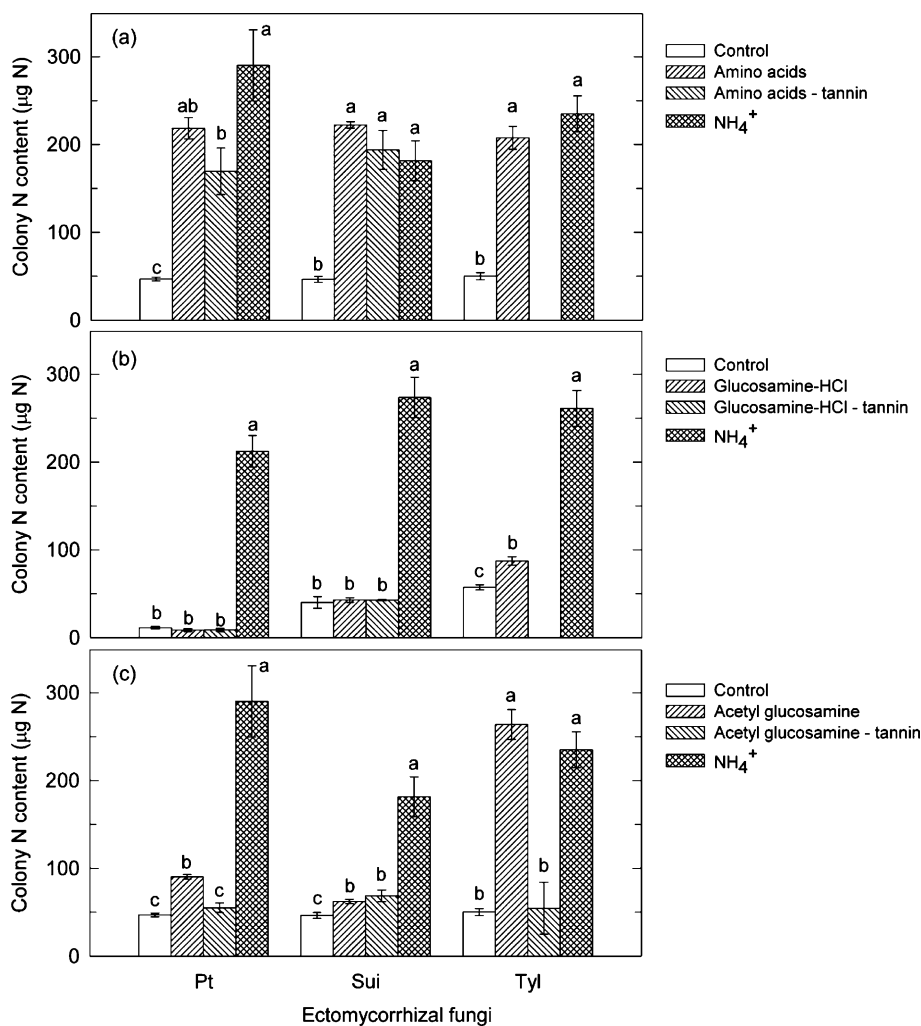

Fig. 3. The mean N content of individual colonies of each of three species of ectomycorrhizal fungi including *P. tinctorius* (Pt), *S. intermedius* (Sui), or *T. felleus* (Tyl) in three experiments. (a) Experiment 3-1: treatments were control, amino acids, amino acids and tannin, or NH_4^+. (b) Experiment 3-2: treatments were control, glucosamine–HCl, glucosamine–HCl and tannin, or NH_4^+. (c) Experiment 3-3: treatments were control, acetyl glucosamine, acetyl glucosamine and tannin, or NH_4^+. In each case, $n=5$. Vertical bars represent ± 1 s.e.m. Within a species, different letters indicate that means are significantly ($p \leq 0.05$) different from each other for each experiment separately. Where there are no data the cultures did not grow.
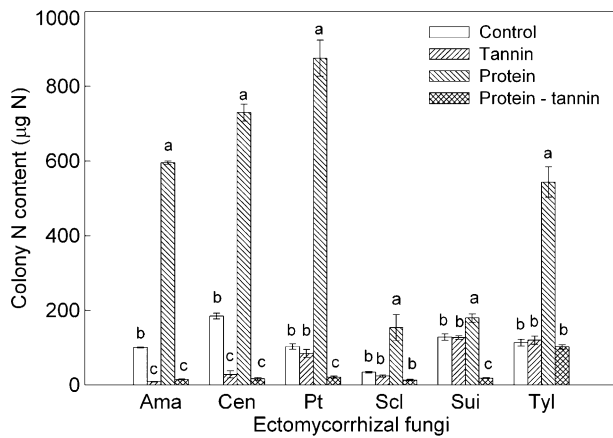
Fig. 4. The mean N content of individual colonies of each of six species of ectomycorrhizal fungi including *A. rubescens* (Ama), *C, geophilum* (Cen), *P. tinctorius* (Pt), *S. citrinum* (Scl), *S. intermedius* (Sui) and *T. felleus* (Tyl) in Experiment 3-4. N treatments included control, tannin, protein, protein and tannin. In each case, $n=8$. Vertical bars represent ± 1 s.e.m. Within a species, different letters indicate that means are significantly ($p \le 0.05$) different from each other.

source, but none of them could utilize protein–tannin complex as an N source (Fig. 4). For *Amanita* (Ama) and *Cenococcum* (Cen), tannin, either alone or in combination with the protein, had a depressive effect on colony N content. For Pt and Sui, tannin had a depressive effect on colony N content only in combination with the protein. For *Scleroderma* (Scl) and Tyl, neither tannin alone nor tannic acid in combination with the protein had a depressive effect.

3.4. Study 4. Potential N sources for saprotrophic fungi

Experiment 4-1. Both *Trichoderma* (Tri) and *Penicillium* (Pen) could obtain N from bovine serum albumen, irrespective of the presence of tannin (Fig. 5a). Tannin alone did not have a depressive effect on the N content of either fungus.

Experiment 4-2. There was clearly something anomalous about the accumulation of N by Tri in this experiment as it had taken up far more N from NH_4^+ in Experiment 4-1. However, both Tri and Pen did obtain a significant amount
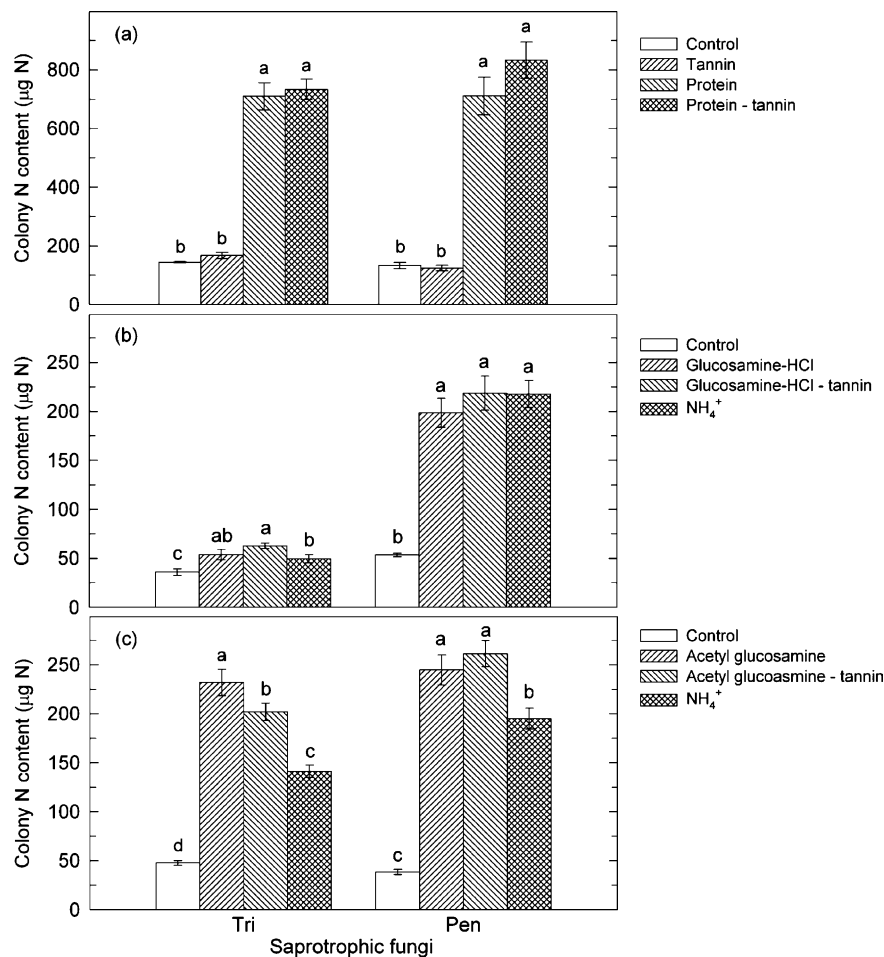

Fig. 5. The mean N content of individual colonies of each of two species of saprotrophic fungi isolated from the forest floor including *Trichoderma* (Tri) and *Penicillium* (Pen) in three experiments. (a) Experiment 4-1: treatments were control, tannin, protein, protein and tannin. (b) Experiment 4-2: treatments were control, glucosamine–HCl, glucosamine–HCl and tannin, or NH_4^+. (c) Experiment 4-3: treatments were control, acetyl glucosamine, acetyl glucosamine and tannin, or NH_4^+. In each case, $n=5$. Vertical bars represent ± 1 s.e.m. Within a species, different letters indicate that means are significantly ($p \le 0.05$) different from each other for each experiment separately.
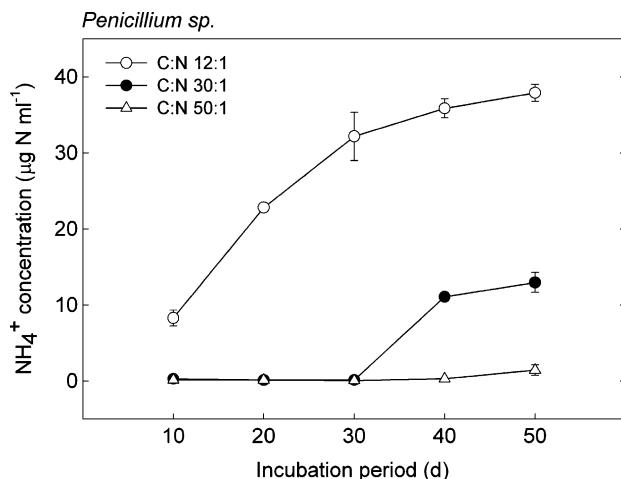
Fig. 6. The mean concentration of NH_4^+ over the course of the 50 d incubation of *Penicillium* in the presence of protein–tannin complex as the only N source at three C:N ratios (Experiment 4-4). Vertical bars represent ± 1 s.e.m. $n = 3$. Where no vertical bars are visible, they are too small to plot behind the symbols.

of N from glucosamine–HCl, irrespective of the presence of tannin (Fig. 5b). Tri accumulated slightly (but significantly) more N with glucosamine–HCl and tannin than with NH_4^+, but Pen accumulated approximately the same amount of N irrespective of N source.

Experiment 4-3. Both Tri and Pen could obtain N from acetyl glucosamine, irrespective of the presence of tannin (Fig. 5c). Both species preferred acetyl glucosamine to NH_4^+.

Experiment 4-4. Pen was able to ammonify the protein–tannin complex, irrespective of C:N ratio (Fig. 6), but ammonification occurred more rapidly the lower the C:N ratio. At 50 d, the dry weight and N content of the colonies was greatest at C:N of 50:1 and least at 12:1, but N concentration was least at 50:1 (Table 2). This indicates that colony growth was limited by C and not by N.

4. Discussion

The availability of N limits plant growth in most temperate forest ecosystems (Gosz, 1981; Aber et al., 1989; Stump and Binkley, 1992). Our vector analysis established that the *P. resinosa* in the plantation from which

Table 2
Mean (s.e.m.) *Penicillium* colony dry weight, N concentration and N content on day 50 of Experiment 4-4

C:N	Dry weight (mg per colony)	N concentration (%)	N content (μg N per colony)
12:1	33.3 (0.6)[c]	3.85 (0.11)[a]	1.28 (0.03)[c]
30:1	63.7 (4.7)[b]	3.54 (0.06)[b]	2.26 (0.12)[b]
50:1	103.7 (3.3)[a]	2.89 (0.13)[c]	3.01 (0.12)[a]

Colonies were grown starting at three different C:N ratios including 12:1, 30:1 and 50:1. The N was supplied as protein–tannin complex. $n = 3$. Different letters indicate that means are significantly ($p \le 0.05$) different from each other.

most of the fungi in this study were isolated (except *P. tinctorius* and *C. geophilum*) were also N limited. When N limits plant productivity and where soils contain high concentrations of organic N, direct access to the organic N by the plant, or indirect access to it via ectomycorrhizal fungi would be beneficial to the plant. Either could increase the competitive ability of the plant relative to other soil microorganisms, and could prevent substantial leaching of mineralized forms of N (Northup et al., 1995). Because plants and their ectomycorrhizal fungi do not have access to all forms of organic N (Stribley and Read, 1980; Abuzinadah et al., 1986; Keller, 1996; Sawyer et al., 2003), we compared *P. resinosa*, ectomycorrhizal fungi and common saprotrophic microfungi in their abilities to access N from a range of organic sources.

Amino acids and proteins may be among the most abundant forms of organic N in the soil (Grov, 1963a,b; Sowden and Ivarson, 1966; Abuarghub and Read, 1988; Turnbull et al., 1996; Schulten and Schnitzer, 1998; Johnsson et al., 1999). Some plant species can use amino acids as a nitrogen source (Miettinen, 1959; Millar and Schmidt, 1965; Chapin et al., 1993). However, while our nonmycorrhizal *P. resinosa* seedlings did respond positively to ammonium, they did not respond to the addition of amino acids, which are among the simplest of all organic N sources in the forest floor. Significant access to organic N by *P. resinosa* seedlings, therefore, would appear to have to be primarily indirect, via their ectomycorrhizal fungi.

The isolates of *Pisolithus*, *Suillus* and *Tylopilus* were capable of using the amino acids as a source of N as readily as NH_4^+. Moreover, all six of the tested ectomycorrhizal fungal isolates were able to obtain N from the protein bovine serum albumen. That other ectomycorrhizal fungi have access to protein and amino acids was demonstrated previously (Melin and Nilsson, 1953; Abuzinadah and Read, 1988; Abuzinadah et al., 1986; Finlay et al., 1992; Chalot et al., 1995; Turnbull et al., 1995, 1996). None of the six tested ectomycorrhizal fungi species were able to obtain N from protein–tannin complex. Bending and Read (1996) also showed that several ectomycorrhizal fungal species had only a very limited capacity to acquire N from protein–tannin complex. Amino sugars are also a potentially important source of N in the forest floor (Johnsson et al., 1999; Milchalzik and Matzner, 1999; Rodionov et al., 2001; Dai et al., 2002; Turrión et al., 2002; Praveen-Kumar et al., 2002; Xu et al., 2003). Because of the large fungal biomass in the forest floor, glucosamine—the building block of chitin, is particularly abundant. Our ectomycorrhizal fungi showed some ability to acquire N from glucosamine, although there was significant variation among species. *Tylopilus* was clearly the best in obtaining N from glucosamine. *Suillus* and *Pisolithus* could only obtain small amounts of N from acetyl glucosamine and had no ability to obtain N from glucosamine–HCl. Obviously the results of our studies are specific to the fungal isolates we tested, and some ectomycorrhizal fungi may have more

saprotrophic traits than the isolates in our studies (Read and Perez-Moreno, 2003). Nonetheless, these results suggest that while the tested isolates of ectomycorrhizal fungi have access to some organic N sources, a sizeable fraction of organic N in the forest floor may not be readily available to them.

Penicillium and *Trichoderma* are among the most frequently isolated microfungi from soils, including forest soils (Russell, 1973; Watanabe, 2002 and references therein). We do not know whether they are the most active saprotrophic fungi in the forest floor. Nevertheless, they do appear to be physiologically equipped for life in the forest floor. Both isolates were easily able to obtain N from protein and even protein–tannin complex. They also readily acquired N from glucosamine, either with or without tannin, and in most cases they acquired more N from glucosamine than from NH_4^+. With respect to obtaining N from protein–tannin complex or glucosamine, then, the isolates of *Trichoderma* and *Penicillium* were both superior to all tested ectomycorrhizal fungi. This is consistent with the suggestion made by Bending and Read (1996) and Koide and Kabir (2001) that some ectomycorrhizal fungi may depend on the activities of saprotrophic fungi to mobilize N from recalcitrant organic compounds. We note, however, that even if ectomycorrhizal fungi have poorer access to certain organic N sources than the tested saprotrophic fungi, over time the ectomycorrhizal fungi might still be able to garner more N from organic N sources than associated microfungi simply by virtue of possessing longer-lived hyphae (Kaye and Hart, 1997). It seems likely that the hyphae of most ectomycorrhizal fungi would be longer lived than the hyphae from saprotrophic microfungi, but this is not now known. Moreover, if ectomycorrhizal fungi possess larger mycelia that are better defended against competitors than saprotrophic microfungi, this could help them to acquire more N from organic N sources than saprotrophic microfungi. However, whether or not the N acquired by ectomycorrhizal fungi benefits their hosts depends on the transfer of N. The propensity to transfer N to host plants may vary among ectomycorrhizal fungal species and cannot be determined from pure culture studies such as ours.

The acquisition of N from organic sources by saprotrophic microfungi such as *Trichoderma* or *Penicillium* would not result in any benefit to the N economies of either ectomycorrhizal fungi or associated host plants without subsequent transfer of the N. Transfer of N from microfungi to ectomycorrhizal fungi might occur via combative interactions, but this has yet to be demonstrated. We have shown, however, that mineralization of organic N by the microfungi can liberate N from recalcitrant organic sources, making it available to ectomycorrhizal fungi. Lindahl et al. (2002) indicated that saprotrophic microfungi cannot readily transport carbon owing to the absence of rhizomorphs. As a consequence, these microfungi can become locally depleted of C, which could promote N

mineralization. Indeed, we showed that N mineralization of protein–tannin by *Penicillium* was immediate at a low C:N ratio (12:1). At progressively higher C:N ratios (30:1 or 50:1), N mineralization was delayed until presumably the reduction in the C:N ratio caused by respiratory loss of CO_2 finally permitted it to occur. While the resultant ammonium would be subject to leaching given sufficient rainfall, the N it contains would be made available to ectomycorrhizal fungi and plant roots, neither of which may have direct access to the organic N source. Moreover, the large surface area provided by ectomycorrhizal fungi may help to efficiently acquire mineral N from the forest floor (Colpaert and van Laere, 1996) before much of it is leached. Indeed, the microcosm experiments of Koide and Kabir (2001) demonstrated that soil saprotrophs and ectomycorrhizal colonization of *P. resinosa* seedlings by *P. tinctorius* could have additive effects on seedling N content. The results of Wu et al. (2003) suggested that the diffusion rates for ammonium might be sufficiently high (when water is not limiting) to make ectomycorrhizal fungi somewhat superfluous. When N leaching is problematic, however, ectomycorrhizal fungi might not be superfluous.

The presence of tannic acid had variable effects on the various ectomycorrhizal fungi. In some cases (Experiments 3-1–3-3), but not in another (Experiment 3-4) tannic acid depressed the growth of Tyl more so than Sui or Pt. Thus it does not seem likely that Tyl is simply more sensitive to tannic acid than the other two fungi. The reason for the variable response among ectomycorrhizal fungal species, and the variation among experiments remains unexplained for the moment. What is clear, however, is that the two microfungi, *Trichoderma* and *Penicillium*, were much less sensitive to the presence of tannic acid, and that tannic acid did in no case prevent them from acquiring N from an organic source.

Based on their capacities to utilize the various organic N sources, we conclude that the saprotrophic microfungal isolates of *Penicillium* and *Trichoderma* may benefit ectomycorrhizal fungi and ectomycorrhizal host plants by mineralizing complex organic N sources. Because of the dependence of mineralization on the C:N ratio, however, our findings predict that saprotrophic microfungi should be of benefit only when C:N ratios are low enough to permit mineralization. This prediction is consistent with a previous finding from our microcosm experiments that ectomycorrhizal colonization of red pine was only beneficial to plant N uptake when C:N ratios were low (Koide and Kabir, 2001). Unlike some wood decaying basidiomycetes, saprotrophic microfungi such as *Penicillium* and *Trichoderma* do not produce rhizomorphs that are capable of translocating carbon over large distances. Thus, they are more likely to become locally carbon limited, which would increase the probability of their mineralizing N (Lindahl et al., 2002). For that reason, saprotrophic microfungi may be important in the N economy of the ectomycorrhizal symbiosis.

Acknowledgements

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